Articles

Expression and Purification of the Extracellular Ligand-Binding Domain of the Atrial Natriuretic Peptide (ANP) Receptor: Monovalent Binding with ANP Induces 2:2 Complexes†

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ABSTRACT: The receptor for atrial natriuretic peptide (ANP) is a type-I transmembrane protein containing an extracellular ligand-binding domain, a single transmembrane sequence, an intracellular kinase-homologous domain, and a guanylate cyclase (GCase) domain. Binding of ANP to the extracellular domain causes activation of the GCase domain by an as yet unknown mechanism. To facilitate studies of the receptor structure and signaling mechanism, we have expressed the extracellular ANP-binding domain of rat ANP receptor (NPR-ECD) in a water-soluble form. NPR-ECD was purified to homogeneity by ANP-affinity chromatography. SDS−PAGE gave a single 61-kDa band, which coincided with a radioactive band obtained by photoaffinity-labeling with $N_{4\alpha}$-azidobenzoyl-^{125}I-ANP(4−28). Edman degradation gave a single amino-terminal sequence expected for the mature protein. Both trifluoromethanesulfonic acid and peptide-*N*-glycosidase F treatments yielded a 50-kDa band, indicating *N*-glycosylation. The molecular mass of 57 725 Da determined by mass spectrometry indicates the carbohydrate content at 16%. NPR-ECD bound ANP with an affinity comparable to that of the full-length receptor. The ligand selectivity of NPR-ECD (in the order ANP > brain natriuretic peptide ≫ C-type natriuretic peptide) was also similar to that of the full-length receptor. HPLC gel filtration of NPR-ECD gave a peak with an apparent mass of 74 kDa. Preincubation with ANP generated a new 150-kDa peak with a concomitant decrease of the 74-kDa peak. This shift in peak positions was ANP concentration-dependent and was complete at the NPR-ECD-to-ANP molar ratio of 1:1, indicating equimolar binding. The change in the apparent native molecular weight from 74 to 150 kDa suggests that binding causes dimerization of the NPR-ECD:ANP complex to yield an [NPR-ECD:ANP]$_2$ complex.

Atrial natriuretic peptide (ANP)[1] is an antihypertensive hormone that is secreted from the atrium of the heart in response to elevated blood pressure. ANP increases salt excretion, dilates blood vessels, and suppresses aldosterone and renin secretion, leading to lower blood pressure and volume (reviews *1* and *2*). These activities of ANP are mediated by specific cell-surface receptors coupled to guanylate cyclase (GCase). The ANP receptor is a type-I transmembrane protein containing an extracellular ANP-binding domain, a single transmembrane sequence, an intracellular kinase-homologous regulatory domain, and a GCase domain (*3*). The mechanism by which binding of ANP to the extracellular domain causes activation of the intracellular GCase domain is not well understood. Transmembrane signaling by the ANP receptor apparently does not require any other accessory proteins (*4*). It has been suggested that, in the basal state, the kinase-homologous domain interacts with the GCase domain and suppresses cyclase activity and that binding of ANP to the extracellular domain releases this suppressive effect, causing elevation of GCase activity (*5*). Alternatively, it has been proposed that ANP binding to the extracellular domain causes binding of ATP to the kinase-homologous domain inside the cell, which, in turn, activates the GCase-domain (*6*). Yet another hypothesis suggests that ANP binding causes the receptor to dimerize and that this dimerization, which results in dimerization of the intracellular GCase domain, leads to manifestation of the GCase activity (*7*, *8*). It has been reported that the ANP receptor in bovine zona glomerulosa membranes exists as a dimer and that one molecule of ANP

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[1] Abbreviations: ANP, atrial natriuretic peptide; GCase, guanylate cyclase; NPR-ECD, extracellular domain of rat ANP receptor type-A; BNP, brain natriuretic peptide; CNP, C-type natriuretic peptide; N_3Bz-^{125}I-ANP, $N_{4\alpha}$-azidobenzoyl ^{125}I-ANP; PCR, polymerase chain reaction; PTH, phenylthiohydantoin; MALDI/TOF, matrix-assisted laser desorption ionization/time-of-flight; hGH, human growth hormone.

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binds a receptor dimer (9). Dimeric and oligomeric forms of ANP receptors have also been observed in cells that overexpress the ANP receptors (*10*, *11*). However, the same studies found that the occurrence of the receptor dimer or oligomers was not dependent on the presence of ANP. Hence, involvement of receptor dimerization or oligomerization, if it occurs under natural conditions, in the mechanism of signal transduction has remained unclear.

The mechanistic studies of ANP receptor signaling to date have been done mostly by deletion mutagenesis and site-directed mutagenesis and have often relied on the use of intact cells, crude cell extracts, or membrane preparations. While these studies have provided critical insights, particularly into the possible roles of the receptor domains in the signaling processes, the lack of isolated and well-defined experimental systems has precluded definitive resolution of the mechanism of signal transduction. Clearly, further studies by more direct and quantitative analyses of the receptor structure and the mode of receptor−ANP interaction are necessary to define the molecular mechanism responsible for signaling. To facilitate studies of the receptor structure and function, we have expressed the extracellular ANP-binding domain of rat ANP receptor (NPR-ECD) in a soluble form by deleting the transmembrane sequence and the intracellular domains. The expressed protein NPR-ECD bound ANP with an affinity comparable to that of the native ANP receptor. NPR-ECD has been purified to homogeneity, and its ligand-binding characteristics and certain molecular properties have been characterized. In contrast to a previous report that suggested a receptor-to-ANP binding stoichiometry of 2:1 (*9*), we found an equimolar binding stoichiometry between the extracellular domain and ANP, and observed evidence for ligand-induced dimerization of the NPR-ECD:ANP complex to form an $[NPR\text{-}ECD:ANP]_2$ complex.

EXPERIMENTAL PROCEDURES

Materials. Rat ANP(1−28), ANP(4−28), and atriopeptin-I (ANP(5−25)) were synthesized as described previously (*12*). Rat brain natriuretic peptide (BNP) and rat C-type natriuretic peptide (CNP) were obtained from Peninsula Laboratories (Belmont, CA). [^{125}I]NaI (carrier-free, 2.4 mCi/nmol) was purchased from Amersham (Arlington Heights, IL). ^{125}I-ANP(4−28) and $N_{4\alpha}$-azidobenzoyl ^{125}I-ANP(4−28) (N_3Bz-^{125}I-ANP(4−28)) were prepared as described (*13*). COS-1 cells were from the American Type Culture Collection (Rockville, MD). The molecular weight standards for size-exclusion chromatography were obtained from Sigma (St. Louis, MO).

Preparation of a cDNA Construct Encoding the Full-Length Rat ANP Receptor. A cDNA clone encoding rat natriuretic peptide receptor A-type in the Bluescript SK$^+$ plasmid was kindly provided by Dr. David L. Garbers of the University of Texas at Dallas. A KpnI−SmaI fragment containing the entire coding sequence of the ANP receptor was excised and cloned into the KpnI−EcoRV sites of the cytomegalovirus promoter-based vector pcDNA3 (Invitrogen, Carlsbad, CA) to obtain a recombinant plasmid designated as pcDNA3-NPR.

Preparation of a cDNA Construct Encoding the Extracellular Domain of the Rat ANP Receptor. A cDNA encoding the extracellular domain of ANP receptor lacking the transmembrane sequence and the intracellular domain was generated by polymerase chain reaction (PCR) using pcDNA3-NPR as the template and the following oligonucleotide primers: a sense-primer corresponding to the T7 promoter sequence in the pcDNA3 vector (5′-GTAATACGACTCAC-TATAGGGC-3′) and an antisense deletion-primer that produced a termination codon immediately after the codon for Asp435 followed by an SpeI restriction site (5′-GGAAAAGTGACTAGTCTTGGTTGC-3′). The PCR product was digested with KpnI and SpeI and cloned into the KpnI and XbaI sites in the pcDNA3 vector. The resulting recombinant plasmid was designated as pcDNA3-NPR-ECD. The truncated receptor cDNA encodes the sequence from the natural initiation Met28 through Asp435 residue. Asn435 is at the carboxyl-terminus of the sequence segment encoded by exon 6 in the genomic DNA sequence (*14*). Exon 6 is the 3′-most exon of the six exons that correspond to the extracellular domain.

Expression of the Full-Length Rat ANP Receptor in COS-1 Cells and Preparation of Cell Membranes. COS-1 cells were cultured in Dulbecco's modified essential medium supplemented with 5% heat-inactivated fetal bovine serum. The cells in 30 15-cm dishes were transfected with pcDNA3-NPR by the DEAE-dextran method (*15*), using 28 μg of the plasmid per dish. One day after transfection, the culture medium was replaced with fresh medium. Three days after transfection, cells were harvested by scraping into Hanks' balanced salt solution containing the following set of protease inhibitors: 50 μg/mL phenylmethanesulfonyl fluoride, 10 μg/mL benzamidine hydrochloride, 10 μg/mL leupeptin, 2 μg/mL aprotinin, 10 μg/mL bacitracin, and 5 mM EDTA. The suspension was centrifuged at 400*g* for 5 min at 4 °C, and the cell pellet was resuspended in 0.25 M sucrose containing the same set of protease inhibitors. The cells were disrupted using a Parr bomb (*16*). The disrupted cell suspension was centrifuged at 1260*g* for 5 min. The supernatant was collected and centrifuged at 30 000*g* for 15 min. The pellet enriched with the cell membranes was resuspended in 50 mM Tris-HCl buffer, pH 7.5, containing 0.15 M NaCl, the above set of protease inhibitors, and 10% (w/v) glycerol. The membrane suspension was aliquoted, quickly frozen in a dry ice−ethanol bath, and stored at −80 °C.

Expression of NPR-ECD in COS-1 Cells. COS-1 cells were transfected with pcDNA3-NPR-ECD using DEAE-dextran as above. After transfection, the cells were cultured for a period of 3 weeks, harvesting the medium twice a week. The culture medium was pooled, and protease inhibitors were added (final concentrations at 0.1 μg/mL aprotinin, 0.1 μg/mL leupeptin, 50 μg/mL phenylmethanesulfonyl fluoride, and 1 mM EDTA). The mixture was then centrifuged at 8000*g* for 30 min to remove cell debris. The supernatant was collected, quickly frozen in a dry ice−ethanol bath, and stored at −80 °C until use.

Purification of the NPR-ECD by ANP-Affinity Chromatography. ANP-agarose affinity gel (0.3 μmol ANP(4−28) immobilized/mL packed gel volume) was prepared using AffiGel-10 (Bio-Rad Laboratories, Hercules, CA) according to the protocol provided by the manufacturer. The culture medium from the transfected COS-1 cells was thawed and filtered through a 0.45-mm Millipore HA filter. The filtrate was applied to a column of ANP-agarose gel (2.4 cm × 5 cm) equilibrated in 50 mM Tris-HCl buffer, pH 7.5,

containing 0.15 M NaCl at a flow rate of 2.5 mL/min at 4 °C. After application, the column was washed with 500 mL of 50 mM Tris-HCl buffer, pH 7.5, containing 0.15 M NaCl, 200 mL of the same buffer containing 1 M NaCl, and 200 mL of the same buffer without NaCl. Elution was carried out with 50 mM Na acetate buffer, pH 5.6, collecting 9-mL fractions in tubes containing 1 mL of 1 M HEPES buffer, pH 7.5. ANP-binding activity was assayed using 1-μL aliquots of each fraction, and the absorption was measured at 280 nm. The NPR-ECD fractions were aliquoted and stored frozen at −80 °C.

Assay of the ANP-Binding Activity. ANP-binding activity associated with cell membrane preparations was measured as previously described (*13*). The binding activity of NPR-ECD secreted in the culture medium was measured as follows. An aliquot of the culture medium (100 μL) was incubated with ^{125}I-ANP(4−28) (200 000 cpm, approximately 0.05 pmol) in the presence and absence of 0.1 μM unmodified ANP(4−28) at 0 °C for 60 min. The mixture was then separated on a column of Sephadex G-50 (3 mL total gel-bed volume) equilibrated in 10 mM sodium phosphate buffer, pH 7.5, containing 0.15 M NaCl, 0.1% bovine serum albumin, and 0.05% bacitracin. The first 1.4-mL fraction contained protein-bound ^{125}I-ANP(4−28). Unbound ^{125}I-ANP was eluted at a volume greater than 1.7 mL.

The ANP-binding activity of the purified NPR-ECD was measured by adding NPR-ECD (typically, 1 ng in 5 μL aliquots) to 45 μL of assay mixture consisting of 50 mM Tris-HCl buffer, pH 7.5, 0.15 M NaCl, 0.1% bovine serum albumin, 0.05% bacitracin, ^{125}I-ANP(4−28) (200 000 cpm), and varying concentrations of unlabeled ANP(4−28). The mixture was incubated at 0 °C for 60 min. Separation of protein-bound and unbound ^{125}I-ANP(4−28) was carried out in the same manner as above. The total binding was generally about 10% of input ^{125}I-radioactivity. Nonspecific binding was generally 10% of the total binding.

Photoaffinity-Labeling and SDS−PAGE. An aliquot of affinity-purified NPR-ECD (10 μg) was incubated with N_3Bz-^{125}I-ANP(4−28) (200 000 cpm, 0.05 pmol) in 20 μL of 0.1 M HEPES buffer, pH 7.5, in the dark at 0 °C. After 1 h, photolysis was carried out using a Spectroline ultraviolet lamp (23 W; Spectronic Instruments, Rochester, NY) at a distance of 10 cm for 5 min. The mixture was then added with SDS−PAGE sample buffer, boiled for 3 min, and electrophoresed in a 10% polyacrylamide precast gel from Novex (San Diego, CA) according to the manufacturer's protocol. The gel was stained with Coomassie-Blue R250. The gel was then dried and exposed to Kodak XAR-5 X-ray film for 2 h. Molecular weight standards used were from Bio-Rad Laboratories.

Deglycosylation Treatment of NPR-ECD. Purified NPR-ECD (5 μg) was treated with varying amounts of peptide-*N*-glycosidase F (EC 3.2.2.11; from *Flavobacterium meningosepticum*, Oxford GlycoSciences, Rosedale, NY) under nondenaturing conditions in 15 μL of 25 mM HEPES buffer, pH 7.5, containing 20 mM EDTA and 0.02% NaN_3 at room temperature for 3 days. A 5-μL aliquot from the incubation mixtures was separated by SDS−PAGE. Chemical deglycosylation of NPR-ECD with trifluoromethanesulfonic acid was carried out according to the method of Edge et al. (*17*).

HPLC Size-Exclusion Chromatography of NPR-ECD. Size-exclusion chromatography of NPR-ECD was performed on a TSK-G3000SW column (0.75 × 30 cm; TosoHaas, Montgomeryville, PA) at room temperature using 20 mM sodium phosphate buffer, pH 7.5, containing 0.15 M NaCl as solvent at a flow rate of 1.0 mL/min. The absorption at 214 nm was monitored. Prior to injection, NPR-ECD (4.2 μM) was incubated with or without varying concentrations of ANP(1−28) in 0.1 M HEPES buffer, pH 7.5, for 1 h at room temperature in a total volume of 25 μL. A 20-μL aliquot of the incubation mixture was injected onto the column.

Additional Methods. The concentration of purified NPR-ECD was determined by amino acid analysis or from the absorption at 280 nm using the ϵ value of 75 600 M^{-1}. Peptide concentrations were determined by amino acid analysis. Amino-terminal sequencing by Edman degradation was performed in an Applied Biosystems Model 470 protein sequencer (Foster City, CA). Analysis by matrix-assisted laser desorption ionization/time-of-flight (MALDI/TOF) mass spectrometry was performed in a Vestec Voyager mass spectrometer (PerSeptive Biosystems, Framingham, MA) at the Protein/Peptide Micro Analytical Laboratory of the California Institute of Technology (Pasadena, CA). The ProMaC 1.5.3 computer program (Applied Biosystems) was used for calculation of the molecular mass.

RESULTS

Expression and Characterization of the Full-Length ANP Receptor. Transfection of COS-1 cells with the recombinant plasmid pcDNA3-NPR encoding the full-length ANP receptor resulted in expression of ANP-binding activity in the cell membrane. Analysis of ANP binding by a competition-binding assay using ^{125}I-ANP(4−28) as the radioactive tracer (Figure 1A) gave a K_d value of 3.1 nM, based on the single-site model. A two-site model did not significantly improve the data fit. Competition-binding with naturally occurring natriuretic peptide isoforms showed the binding selectivity in the order ANP > BNP ≫ CNP. Atriopeptin-I, a truncated ANP with reduced biological activity, showed weak affinity. Photoaffinity-labeling with the membranes from the transfected COS-1 cells using N_3Bz-^{125}I-ANP(4−28) gave a single 126-kDa band in SDS−PAGE (Figure 2A, lane 3). Inclusion of unmodified ANP(4−28) eliminated labeling of the 126-kDa band (Figure 2A, lane 4), indicating that the labeling was specific. The cells transfected with the pcDNA3 vector alone showed no significant binding with ANP, either in the membranes or in the culture medium (data not shown).

Expression, Purification, and Characterization of NPR-ECD. The extracellular ANP-binding domain of the receptor was expressed in COS-1 cells by transfecting them with pcDNA3-NPR-ECD. ANP binding activity was found in the culture medium, and no measurable binding activity was found with the membranes. Binding activity in the medium reached a maximum level 4−5 days after transfection. This level of expression of the activity was maintained throughout the second week and then gradually decreased. Photoaffinity-labeling with an aliquot of the culture medium by N_3Bz-^{125}I-ANP(4−28) yielded a single, radiolabeled 61-kDa band in SDS−PAGE (Figure 2A, lane 1). Inclusion of unmodified ANP(4−28) eliminated the 61-kDa band (Figure 2A, lane 2).

NPR-ECD was purified by ANP-affinity chromatography from the culture medium of the transfected COS cells (Figure

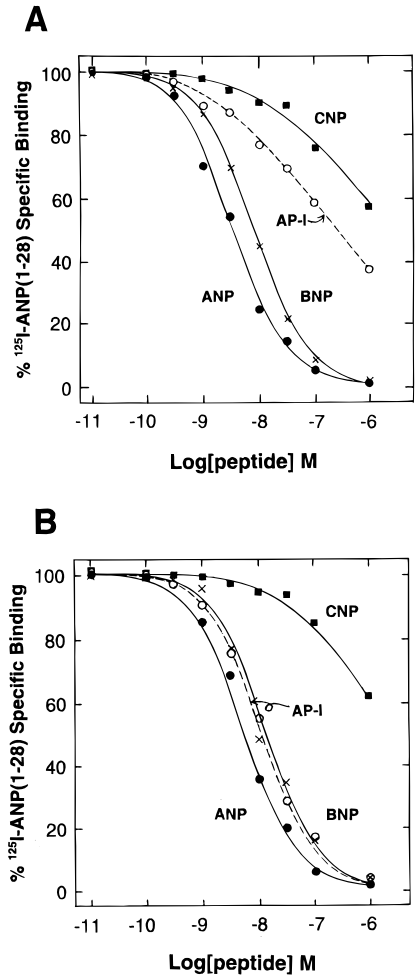

FIGURE 1: Competition-binding assays of full-length rat ANP receptor and the extracellular domain expressed in COS cells (A) The membranes from COS cells expressing the full-length ANP receptor were incubated with varying concentrations of ANP(4−28) (●), BNP (×), CNP (■), or atriopeptin-I (○) in the presence of ^{125}I-ANP(4−28), as described in Experimental Procedures. (B) Binding of purified NPR-ECD was assayed in the same manner.

3). Culture medium collected from 30 dishes (15-cm diameter) over a period of 3 weeks in a total volume of typically 5 L was applied to a column of ANP-agarose. After the column was washed, the bound material was eluted with 0.05 M sodium acetate buffer, pH 5.6. The ANP-binding activity appeared in a peak coinciding closely with an absorption peak at 280 nm. Generally, 0.5−2 mg of NPR-ECD was obtained.

Purified NPR-ECD (10 μg) was photoaffinity labeled with N_3Bz-^{125}I-ANP(4−28) and separated by SDS−PAGE (Figure 2B). Staining with Coomassie Blue gave a single band at the position corresponding to a molecular mass of 61 kDa. Autoradiography of the same gel gave a band that coincided with the stained band. These results clearly demonstrate the identity and the purity of isolated NPR-ECD.

Edman degradation of NPR-ECD yielded a single amino acid sequence, Ser-Asp-Leu-Thr-Val-Ala-Val-Val-Leu-Pro-Leu-Thr-Xxx-Thr-Ser-Tyr-Pro-Trp-Ser-Trp− (Table 1). No other sequence was detected, supporting the homogeneity of the preparation. This finding also indicates that the expressed protein was uniformly processed during synthesis and secretion. The amino terminal sequence of NPR-ECD was consistent with the sequence of the mature ANP receptor

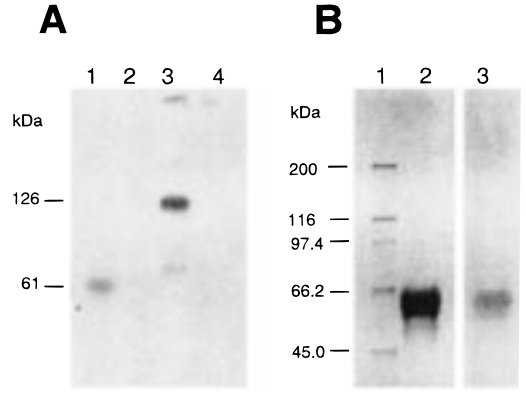

FIGURE 2: SDS−PAGE of full-length rat ANP receptor and the extracellular domain expressed in COS cells. (A) Photoaffinity labeling experiments with N_3Bz-^{125}I-ANP(4−28) and the culture medium from COS cells expressing NPR-ECD (lanes 1 and 2) and with the membranes from cells expressing the full-length receptor (lanes 3 and 4). Even-numbered lanes show the results of control experiments performed in the presence of 0.1 mM ANP(4−28). (B) Affinity-purified NPR-ECD was photoaffinity-labeled and separated by SDS−PAGE. Key: lane 1, standards, lane 2, stained with Coomassie Blue, lane 3, detected by autoradiography.

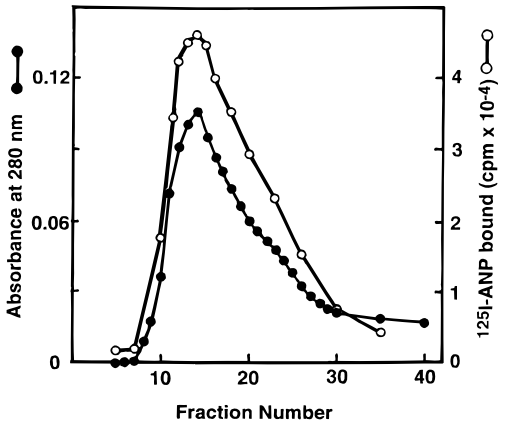

FIGURE 3: Purification of NPR-ECD by ANP-affinity chromatography. The culture medium collected from the transfected COS cells was applied to a column of ANP-agarose gel. After being washed, the bound material was eluted with 50 mM Na acetate buffer, pH 5.6. Absorption at 280 nm was monitored. Specific binding of ^{125}I-ANP(4−28) observed with a 1-μL aliquot of each fraction is plotted.

Table 1: Edman Degradation of NPR-ECD

cycle no.	PTH amino acid	yield (pmol)[a]	cycle no.	PTH amino acid	yield (pmol)[a]
1	Ser	23.3	11	Leu	16.9
2	Asp	36.7	12	Thr	12.7
3	Leu	30.0	13	Asn	0.0
4	Thr	24.8	14	Thr	12.9
5	Val	27.2	15	Ser	5.7
6	Ala	27.6	16	Tyr	10.0
7	Val	27.2	17	Pro	6.2
8	Val	28.8	18	Trp	0.7
9	Leu	26.3	19	Ser	2.8
10	Pro	14.4	20	Trp	1.2

[a] The values for the yields of PTH amino acids have been corrected for the backgrounds.

that was suggested earlier on the basis of the cDNA sequence (*3*).

The molecular mass of NPR-ECD was measured by MALDI/TOF mass spectrometry using horse heart cytochrome *c* and bovine serum albumin as the internal standards

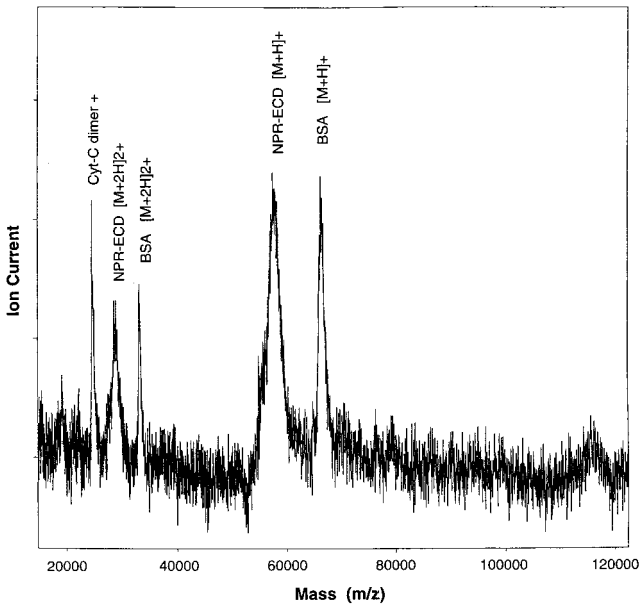

FIGURE 4: Mass spectrum of NPR-ECD obtained by matrix-assisted laser desorption ionization/time-of-flight mass spectrometry. The peaks of singly charged and doubly charged ions ($[M + H]^+$ and $[M + 2H]^{2+}$, respectively) of NPR-ECD are identified in the spectrum, along with those of bovine serum albumin (BSA) and singly charged ion of cytochrome *c* dimer (Cyt-C dimer). Bovine serum albumin and cytochrome *c* were added to purified NPR-ECD and were used as the internal standards.

(Figure 4). A mass value of 57 633 ± 42 Da was obtained from the measurement of singly charged ions and 57 817 ± 96 Da from doubly charged ions. An estimated mass value of 57 725 Da was obtained by averaging the two values. The observed mass for NPR-ECD was considerably greater than the mass of 48 374 Da (average mass) calculated for the polypeptide consisting of residues 1−435 and containing 3 disulfide linkages (unpublished data). The larger observed mass value apparently is the result of glycosylation (see below). The peaks observed for NPR-ECD were considerably broader compared with those of cytochrome *c* and serum albumin, presumably reflecting microheterogeneity in glycosyl moieties.

Chemical deglycosylation with trifluoromethanesulfonic acid yielded a 50-kDa band in SDS−PAGE (Figure 5A). Treatment with peptide-*N*-glycosidase F also gave a 50-kDa band (Figure 5B). These results indicate that NPR-ECD contains oligosaccharides (approximately 16 wt %), mostly in N-linked forms. Edman degradation (Table 1) in cycle 13 gave no increase in any phenylthiohydantoin (PTH) amino acid. The cDNA sequence encodes Asn for position 13, which constitutes a part of the consensus sequence Asn-Xxx-Thr/Ser for protein N-glycosylation. Absence of PTH-Asn at position 13 thus indicates that residue Asn13 is indeed glycosylated.

Ligand Specificity and ANP-Binding Stoichiometry of NPR-ECD. Binding of the purified NPR-ECD was studied by competition-binding. Analysis of ANP(4−28) binding gave a K_d of 6.0 nM based on a single-site model (Figure 1B). A two-site model did not improve data fit. Competition-binding with natriuretic peptide isoforms showed the binding selectivity of NPR-ECD in the order ANP > BNP ≫ CNP, which was identical to that found with the full-length ANP receptor in COS-1 cell membranes (Figure 1A). In contrast,

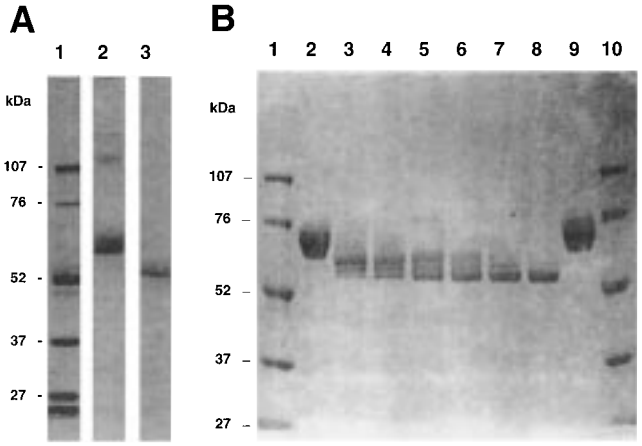

FIGURE 5: Deglycosylation of NPR-ECD. (A) Chemical deglycosylation of NPR-ECD with trifluoromethanesulfonic acid (lane 1, protein standards; lane 2, untreated NPR-ECD; lane 3, NPR-ECD deglycosylated with trifluoromethanesulfonic acid). (B) NPR-ECD (5 μg) was incubated without (lanes 2 and 9) or with varying amounts of peptide-*N*-glycosidase F (0.1, 0.2, 0.5, 1.0, 2.0, and 4.0 units; lanes 3−8) at room temperature for 3 days. Lanes 1 and 10 are protein standards.

atriopeptin-I, which had weak affinity to the full-length receptor (Figure 1A), bound to NPR-ECD with high affinity (Figure 1B).

To determine the binding stoichiometry between NPR-ECD and ANP, NPR-ECD at 4.2-μM concentration was incubated with and without varying concentrations of ANP-(1−28), and the mixture was then separated by HPLC size-exclusion chromatography (Figure 6A). Without ANP(1−28), NPR-ECD gave a single peak eluting at the position corresponding to an apparent molecular mass of 74 kDa, based on the calibration curve shown in Figure 6B. Incubation of NPR-ECD with increasing concentrations of ANP resulted in the appearance of a new peak having an apparent mass of 150 kDa and in a concomitant decrease of the 74-kDa peak. The size of the 150-kDa peak increased roughly in proportion to the concentration of added ANP through 5.0-μM concentration. At ANP concentrations above 5.0 μM, a peak of unbound ANP(1−28) was detected at the elution volume of 13.2 mL. Thus, the shift from the 74-kDa peak to the 150-kDa peak in response to the addition of ANP reached completion at the ANP-to-NPR-ECD molar ratio of approximately 1:1, indicating an equimolar binding stoichiometry. The large change in the apparent mass from 74 to 150 kDa indicates that binding causes dimerization of the 1:1 NPR-ECD:ANP complex, yielding an $[NPR\text{-}ECD:ANP]_2$ complex. At any of the ANP concentrations tested, ranging from 0.2 to 100 μM (or 0.05-fold to 25-fold molar equivalent to NPR-ECD), no new peak other than the 150-kDa peak was found, suggesting that only the $[NPR\text{-}ECD:ANP]_2$ complex was formed as a stable complex.

DISCUSSION

To facilitate studies of the ANP receptor structure and signaling mechanism, we have expressed the extracellular ligand-binding domain of rat ANP receptor in a soluble form. The expressed protein NPR-ECD largely retained the binding affinity and ligand specificity of the native ANP receptor. NPR-ECD has been purified from the culture medium of the transfected cells by a single step of ANP-affinity

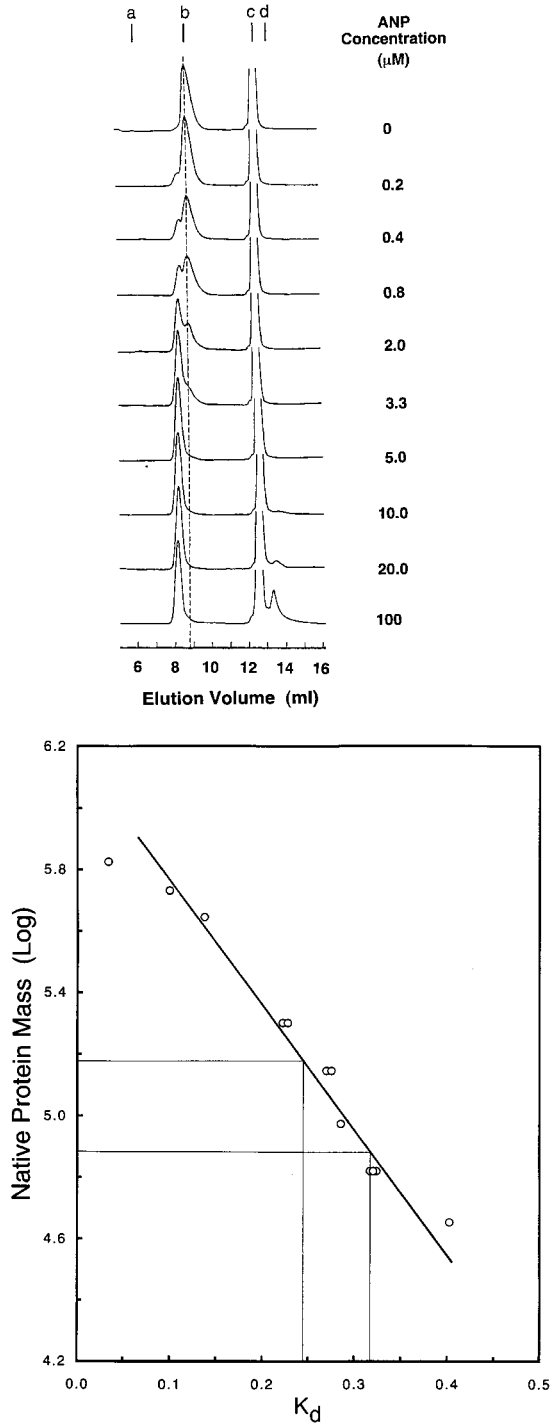

FIGURE 6: Binding of NPR-ECD with ANP(1−28) analyzed by size-exclusion chromatography on a TSK-G3000SW column. (A) Top: NPR-ECD at a concentration of 4.2 μM was incubated with or without ANP(1−28) at the concentrations indicated on the right at room temperature for 1 h, and the mixture was then injected. The elution positions of Blue Dextran, NPR-ECD, buffer salts, and ANP(1−28) are shown by arrows a−d, respectively. (B) Bottom: Calibration curve generated by chromatography of standard proteins, thyroglobulin (669 kDa), β-galactosidase (540 kDa), apoferritin (443 kDa), β-amylase (200 kDa), yeast alcohol dehydrogenase (140 kDa), phosphorylase-b (94 kDa), bovine serum albumin (66 kDa), and ovalbumin (45 kDa). The logarithm of the native protein mass was plotted against the distribution coefficient K_d ($K_d = (V_e - V_0)/(V_t - V_0)$, in which V_e, V_0, and V_t are the elution volume of a standard protein, column void volume, and column total volume, respectively). The K_d values obtained for unbound NPR-ECD and ANP-bound NPR-ECD were 0.32 and 0.24, which gave apparent masses of 74 and 150 kDa, respectively.

chromatography (Figure 3). The homogeneity of the preparation was confirmed by observing a single Coomassie Blue-stained band in SDS−PAGE, which coincided with the radioactive band obtained by photoaffinity-labeling with N_3-Bz-^{125}I-ANP(4−28) (Figure 2). The single amino-terminal sequence obtained by Edman degradation also supports homogeneity of the preparation. NPR-ECD had a molecular mass of 57 725 Da as estimated by MALDI/TOF mass spectrometry and contained approximately 16% (w/w) oligosaccharides, mostly in N-linked forms.

NPR-ECD bound its natural ligand ANP with an affinity similar to that observed with the naturally occurring ANP receptor (*13*) or the full-length receptor expressed in COS-1 cell membranes (Figure 1). NPR-ECD showed binding selectivity toward natriuretic peptide isoforms in the order ANP > BNP ≫ CNP. Such selectivity is typical of A-type natriuretic peptide receptors (*18*−*20*). The full-length ANP receptor expressed in the COS-1 cell membranes showed essentially the same selectivity as the native receptor. These results together indicate that NPR-ECD largely retained the ligand-binding characteristics of the native receptor. NPR-ECD expressed and purified with the ligand-binding properties similar to those of the native receptor provides useful material for investigation of the structure of the extracellular domain and its function in signal transduction.

The stoichiometry of ligand binding has direct implication in the mechanism of signal transduction. The availability of purified NPR-ECD allowed accurate determination of its concentration and direct analysis of the binding stoichiometry with ANP. NPR-ECD at a fixed concentration was incubated with increasing concentrations of ANP(1−28), and binding was monitored by rapid separation by HPLC size-exclusion chromatography (Figure 6). Chromatography of NPR-ECD alone gave a single 74-kDa peak. Incubation of NPR-ECD with ANP gave rise to both a new 150-kDa peak, representing ANP-bound NPR-ECD, and a concomitant decrease of the 74-kDa peak. This shift from the 74-kDa peak to the 150-kDa peak was linearly correlated with the concentration of ANP added to the incubation and reached completion at the ANP-to-NPR-ECD molar ratio of 1:1. These results indicate an equimolar binding stoichiometry between NPR-ECD and ANP. The apparent mass of 150 kDa for the bound complex also indicates that, upon binding, the NPR-ECD:ANP complex undergoes intermolecular association to form a dimeric complex, $[NPR\text{-}ECD:ANP]_2$.

Previously, Rondeau et al. (*9*) reported the binding stoichiometry of the receptor to ANP to be 2:1 and suggested that the ANP receptor forms a 2:1 receptor-to-ANP complex as its signal transducing complex. In their experiments using bovine zona glomerulosa membrane preparations, the binding stoichiometry was estimated for both membrane-bound and detergent-solubilized ANP receptor by taking the ratio between the receptor density estimated by saturation-binding assay and the amount of the receptor protein measured by radioimmunoassay using antireceptor peptide antibodies. However, it is possible that the density of binding sites and the amount of the receptor protein estimated using two different assay techniques have yielded an unreliable correlation. These investigators also described a cross-linking experiment that used an ANP analogue containing a photoactivatable group at both its amino- and carboxyl-terminal ends. The photocross-linking reaction yielded an immuno-

stained, high-M_r band in SDS−PAGE. This high-M_r band was suggested to represent a receptor dimer cross-linked by the affinity reagent. However, no direct evidence was presented to show that the immuno-stained band indeed was that of the receptor dimer. In contrast to those studies with the ANP receptor in bovine adrenal membranes (*9*), the present study with the truncated extracellular domain of rat brain ANP receptor, which was purified and quantitated by amino acid analysis, clearly showed monovalent binding between the receptor extracellular domain and ANP.

The ANP receptor shares its overall molecular topology with growth hormone receptors and cytokine receptors, where a single transmembrane sequence links the extracellular ligand-binding domain and the intracellular effector enzyme domain. Among such receptors, the hGH receptor and the erythropoietin receptor form a 2:1 receptor-to-ligand complex as their signal transducing complex (*21*−*23*). The extracellular domain of these receptors, when incubated with varying concentrations of the respective ligand, was found to generate 1:1 and 2:1 protein-to-ligand complexes, which could be separated by size-exclusion chromatography (*21*, *23*). In contrast with NPR-ECD, only the 150-kDa peak, which apparently represents the $[NPR\text{-}ECD:ANP]_2$ complex, was observed at any of the ANP concentrations tested; these ranged from 0.05-fold to 24-fold over the NPR-ECD concentration in the incubation. These results indicate that only the 2:2 complex, but not the 2:1 complex, was formed between NPR-ECD and ANP.

In contrast to the hGH receptor and the erythropoietin receptor, the receptors for epidermal growth factor (*24*) and granulocyte-colony stimulating factor (*25*) have been shown to form a 1:1 complex with the ligand, which then undergoes dimerization to form a 2:2 signaling complex. The extracellular domain of these receptors forms a corresponding 2:2 protein-to-ligand complex in vitro that can be separated by size-exclusion chromatography (*24*, *25*). The behavior of NPR-ECD was remarkably similar to that of the extracellular domain of the latter two receptors. These findings suggest that signal transduction by the ANP receptor may involve a mechanism similar to those of the epidermal growth factor receptor and granulocyte-colony stimulating factor receptor, whereby the receptor bound with its ligand at 1:1 stoichiometry undergoes dimerization to form a 2:2 signal transducing complex. Interestingly, during the course of this study, we noticed that injection of higher concentrations of NPR-ECD, without ANP, into the size-exclusion column yielded single peaks eluting near the position corresponding to an apparent mass of approximately 138 kDa (data not shown), suggesting spontaneous self-association of NPR-ECD to form a homodimer. Thus, it appears that NPR-ECD is in a rapid and concentration-dependent dynamic equilibrium between its monomer and dimer states, even in the absence of ANP. Importantly, this finding indicates that receptor dimerization alone is not a sufficient mechanism for receptor activation.

The extracellular domains of the ANP receptor, the BNP receptor, and the ANP clearance-receptor have previously been expressed as fusion proteins with the Fc portion of human IgG-γ_1 (*26*). These fusion proteins were found to have a binding specificity similar to those of the native receptors and were used to develop solid-phase screening assays for receptor ligands. In fused proteins, however, the conformation or the behavior of the expressed domain is often influenced by the fused exogenous polypeptide. For example, the extracellular domain-IgG fusion proteins constructed in those studies were secreted as disulfide-linked homodimers, which would not be useful for examining the behaviors of the extracellular domain, such as self-association and conformational changes. NPR-ECD expressed free of exogenous, fused polypeptide is likely to have the least structural interference and is useful for investigation of the structure and function relationships.

A truncated peptide atriopeptin-I, or ANP(5−25), has weak natriuretic and vasorelaxant activities (*27*, *28*) and is a poor ligand for the native ANP receptor (*13*). Consequently, atriopeptin-I showed low affinity to the full-length receptor expressed in COS-1 cell membrane (Figure 1A). In contrast, atriopeptin-I bound to NPR-ECD with high affinity (Figure 1B). These results suggest that the truncation of the receptor polypeptide caused a subtle, but yet distinct, change in the binding-site structure. Previously, we observed that incubation of bovine adrenal membranes at a slightly acidic pH caused a limited proteolytic cleavage of the ANP receptor near the transmembrane sequence, yielding a 65-kDa, ANP-binding polypeptide fragment (*29*, *30*). This cleaved receptor also bound atriopeptin-I with high affinity. In addition, this proteolytic cleavage was found to cause inactivation of the membrane-bound GCase activity. While the mechanisms responsible for these changes in the ligand selectivity and GCase activity are not known, these findings together suggest that the structure near the transmembrane sequence is conformationally as well as functionally related to the binding site and may play a critical role in transmembrane signaling.

In summary, the extracellular ligand-binding domain of the ANP receptor has been expressed in an active form and purified by ANP-affinity chromatography in milligram quantities. The expressed protein largely retained the structural and functional characteristics expected for the extracellular domain of the ANP receptor. The expressed protein NPR-ECD showed 1:1 binding with ANP, and binding of ANP caused dimerization to form an $[NPR\text{-}ECD:ANP]_2$ complex. These results establish that ligand-induced receptor dimerization is an intrinsic property of the extracellular ligand-binding domain of the ANP receptor. The purified ANP receptor ligand-binding domain made available by the present study will be useful for direct and quantitative studies of the structure and function of the extracellular domain, such as analyses of the ligand-binding mechanism, ligand-dependent self-association, ligand-induced conformational changes, and the 3-dimensional structure. Such information is necessary to understand the molecular mechanism of transmembrane signal transduction by the ANP receptor.

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